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THE EFFECTS OF QUATERNARY GLACIATIONS ON
THE GENETIC STRUCTURE OF *SPELEOMANTES*
STRINATII (AELLEN, 1958)

(AMPHIBIA, PLETHODONTIDAE)

INTRODUCTION

The alternation of cold and warm stages of glacial and interglacial periods during the Pleistocene had a strong impact on the distribution and evolution of vegetation and associated faunal species (COMES & KADEREIT 1998; HEWITT 2000). One of the animal genera strongly influenced by the succession of glacial and interglacial stages has been the plethodontid salamander *Speleomantes*, due to its particular biological features. Indeed these salamanders usually hatch completely metamorphosed from eggs, lack an aquatic larval stage, are lungless with cutaneous and bucco-pharyngeal respiration and are strongly associated with underground retreats, offering a steady cold and moist environment (SALVIDIO *et alii* 1994; CIMMARUTA *et alii* 1999; LANZA 1999). These features, coupled with low vagility and restricted home ranges, make *Speleomantes*' geographic distribution strongly affected by climatic variations.

In particular, the species *Speleomantes strinatii* has a relatively wide range, compared with those of the congeneric species, comprising north-western Italy and south-eastern France. In these zones

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the climatic variations of the Quaternary had great influence on the evolutionary history of local populations because of the uneven local orography. The genetic diversity of this species, investigated by means of allozyme electrophoresis, has been correlated with the paleoclimatic events of the species range, to understand the microevolutionary mechanisms leading to its extant genetic structure. A number of subspecies has been described on morphological basis within this species (see LANZA *et alii* 1995: p.53) but their validity has been recently challenged based on genetic ground (NASCETTI *et alii* 1996). The data obtained here on the genetic-geographic structure of *S. strinatii* have been used to reanalyse the systematics of these questioned taxa.

MATERIALS AND METHODS

About 400 specimens of *S. strinatii*, collected from 29 populations scattered through the whole range of the species have been studied (Tab. 1).

The techniques used for multilocus electrophoresis are those reported in LANZA *et alii* (2005) and are described in detail in LANZA *et alii* (1995). Following these procedures with minor modifications, the following 33 putative loci have been scored: α -Gpdh, Ldh-1, Ldh-2, Hbdh, Mdh-1, Mdh-2, Mdhp-1, Mdhp-2, Idh-1, Idh-2, 6-Pgdh, Gapdh, NADH-dh, Sod-1, Sod-2, Np, Aat-1, Aat-2, Ck, Adk, Est-4, Pep-C, Ped-D, Lap, Ada-1, Ada-2, Ca-2, Ca-3, Fum, Mpi, Gpi, Pgm-1, Pgm-2. They included about one third of fast and two thirds of slow evolving loci, *sensu* SARICH (1977) and KOEHN & EANES (1978).

The genetic variability of populations was evaluated using the following parameters: overall number of alleles per population (A); percentage of polymorphic loci (P) following 99% (P_{99}) and 95% (P_{95}) criteria and mean expected (H_e) and observed (H_o) heterozygosity per population. The statistical significance of departures from the Hardy-Weinberg equilibrium was estimated using χ^2 test.

Genetic distances (D_{Nei} and D_{Rogers}) have been calculated according to both NEI's (1972) and Rogers' (WRIGHT 1978) methods. Calculations were performed using BIOSYS-1 computer program (SWOFFORD & SELANDER 1989). Times of evolutionary divergence

(t) were calculated using the formula proposed by NEI (1975) when $D_{Nei} < 1$: $t = 5 \cdot 10^6 \cdot D_{Nei}$.

A principal component analysis (PCA) was carried out using SYSTAT 5.2 (WILKINSON *et alii* 1992). The PCA was based on a matrix of the allele frequencies of polymorphic loci, after removing alleles with frequencies lower than 10%.

Table 1 - Code number of *Speleomantes strinatii* samples listed with the collecting sites and their province and region.

Number	Collecting sites	Province	Region/Country
1	Pietra di Vasca Mt.	Genoa	Liguria
2	Eastern Slope of Groppi Mt.	Genoa	Liguria
3	Environs of Carro and Ponte S. Margherita	La Spezia	Liguria
4	Pù Mt., in the Rio Frascaresse Valley	Genoa	Liguria
5	Near Cassagna	Genoa	Liguria
6	Cave "Tana de Strie"	La Spezia	Liguria
7	Cave "Tana da Cruxetta"	La Spezia	Liguria
8	Near Maissana and SW slope of Baralucco Mt.	La Spezia	Liguria
9	Near Codolo	Massa Carrara	Tuscany
10	Near Bardi	Parma	Emilia Romagna
11	Near S. Pietro di Novella and Rapallo	Genoa	Liguria
12	Near Scaglia, Bargagli	Genoa	Liguria
13	Near Cartasegna	Alessandria	Piemonte
14	Near Isoverde	Genoa	Liguria
15	Near Millesimo	Savona	Liguria
16	Near Roveirola, N slope of Bric Cornavento	Savona	Liguria
17	Near Finale Ligure	Savona	Liguria
18	Near Gavone	Savona	Liguria
19	Near Toirano	Savona	Liguria
20	Left bank of the Rio Roburentello stream	Cuneo	Piedmont
21	Near Roaschia and near Rivoera	Cuneo	Piedmont
22	S. Bartolomeo Hill, S slope	Imperia	Liguria
23	Near Caravonica	Imperia	Liguria
24	Downstream Tenarda dam	Imperia	Liguria
25	Near Rocchetta Nervina	Imperia	Liguria
26	Near Tenda		France
27	Near Luceram		France
28	Between Peille and St. Martin de Peille		France
29	Near Aspremont		France

RESULTS

Eleven loci out of the 33 studied were monomorphic, showing the same allele in all the populations sampled: *Idh-2*, *NADH-dh*, *Np*, *Sod-1*, *Sod-2*, *Aat-2*, *Adk*, *Pep-C*, *Lap*, *Fum*, *Mpi*. The remaining 22 loci showed from two to four alleles per population. The pattern of allele frequencies at many loci concurred in showing a higher genetic variation in western populations, while samples from the eastern part of the range were genetically more homogeneous. This pattern is confirmed by the distribution of the private alleles (i.e., alleles found in a single population; NEEL 1973): 15 over 18 private alleles where in the samples from the western part of the

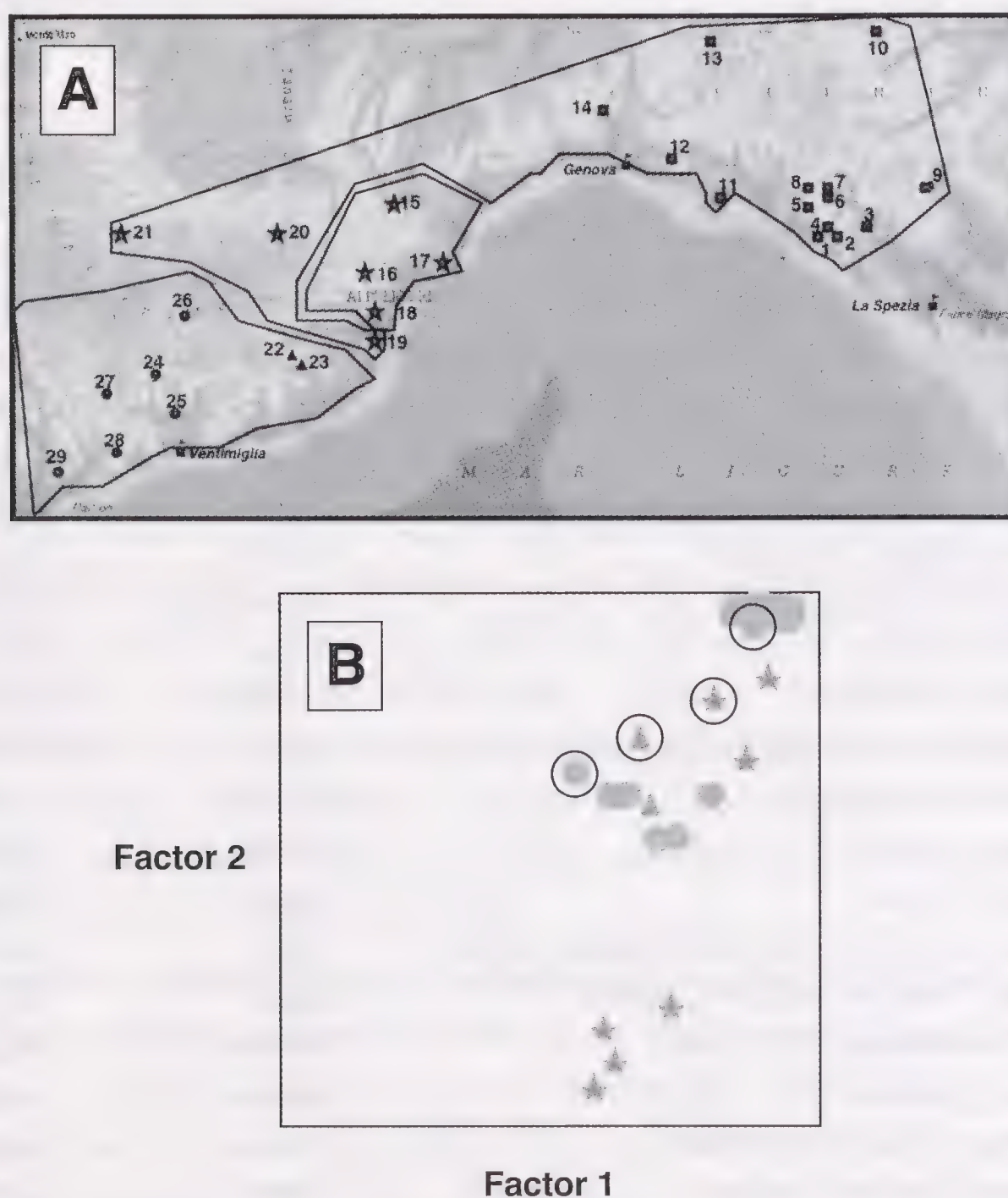


Fig. 1 - A) Geographical distribution of the studied populations, symbols refer to the subspecies to which they were ascribed on morphological ground. B) PCA based on allele frequencies, the percentage of the total variance explained is 88.7 (80.6 is explained by Factor 1). The *terra typica* of each subspecies is circled.

■ (squares) *S. s. ligusticus*, ★ (stars) *S. s. argentatus* and "nameless" samples from Finalese (15-18), ▲ (triangles) *S. s. bonzanoi*, ● (circles) *S. s. strinatii*.

Table 2 - Parameters of genetic variability in the 29 studied populations of *S. stri-natii*. Standard errors are in parentheses.

Population	$\underline{A}$	P_{99}	P_{95}	H_o	H_e
1.	1.1	12.1	6.1	0.009 (0.005)	0.011 (0.006)
2.	1.1	9.1	9.1	0.047 (0.029)	0.035 (0.020)
3.	1.1	6.1	6.1	0.027 (0.019)	0.023 (0.016)
4.	1.1	9.1	9.1	0.022 (0.013)	0.022 (0.013)
5.	1.1	6.1	3.0	0.010 (0.008)	0.009 (0.007)
6.	1.1	6.1	3.0	0.007 (0.006)	0.007 (0.005)
7.	1.1	6.1	6.1	0.018 (0.012)	0.022 (0.016)
8.	1.1	12.1	12.1	0.034 (0.020)	0.028 (0.015)
9.	1.1	6.1	6.1	0.014 (0.010)	0.019 (0.015)
10.	1.0	3.0	3.0	0.013 (0.013)	0.011 (0.011)
11.	1.1	9.1	9.1	0.031 (0.018)	0.037 (0.022)
12.	1.1	9.1	9.1	0.035 (0.022)	0.030 (0.018)
13.	1.1	9.1	9.1	0.025 (0.016)	0.027 (0.017)
14.	1.1	9.1	9.1	0.027 (0.017)	0.024 (0.015)
15.	1.1	9.1	9.1	0.036 (0.022)	0.031 (0.018)
16.	1.2	21.2	21.2	0.079 (0.030)	0.085 (0.031)
17.	1.2	18.2	18.2	0.062 (0.028)	0.056 (0.024)
18.	1.2	15.2	15.2	0.052 (0.025)	0.050 (0.024)
19.	1.4	33.3	18.2	0.079 (0.028)	0.077 (0.027)
20.	1.1	12.1	12.1	0.041 (0.022)	0.043 (0.023)
21.	1.1	6.1	6.1	0.020 (0.015)	0.021 (0.015)

Table 2 - (Continued) Parameters of genetic variability in the 29 studied populations of *S. strinati*. Standard errors are in parentheses.

Population	<u>A</u>	P_{99}	P_{95}	H_o	H_e
22.	1.4	36.4	30.3	0.093 (0.027)	0.125 (0.036)
23.	1.3	27.3	27.3	0.108 (0.034)	0.108 (0.034)
24.	1.4	30.3	21.2	0.088 (0.030)	0.091 (0.031)
25.	1.3	24.2	24.2	0.093 (0.031)	0.096 (0.031)
26.	1.2	24.2	24.2	0.091 (0.032)	0.085 (0.030)
27.	1.2	18.2	18.2	0.081 (0.032)	0.075 (0.030)
28.	1.3	21.2	15.2	0.048 (0.020)	0.065 (0.026)
29.	1.3	27.3	24.2	0.077 (0.026)	0.089 (0.030)

range (samples 15-29), while only three have been found in the 14 easternmost populations. Moreover, all the private alleles with frequencies higher than 10% have been found in western populations. The higher genetic variation of westernmost populations is also evidenced by the values of genetic variability parameters (Tab. 2). In the easternmost populations (1-14) the averaged expected heterozygosity was 0.022 (range 0.007-0.037) and the percentage of polymorphic loci (P_{99}) had a mean value of 8.0% and a range of 3-12.1%. The values observed for the samples 15-21 were higher, with mean values of H_e reaching 0.052 (range 0.021-0.085) and P_{99} between 6.1-33.3 (mean value 16.5). The western populations (samples 22-29) show the highest levels of genetic variability, with heterozygosity values ranging between 0.065 and 0.125 and a percentage of polymorphic loci from 18.2 to 36.4. Mean values of these parameters were H_e =0.092 and P_{99} =26.1, two to four times those recorded for the other populations.

The PCA evidences three main groups of samples (fig. 1B). The group in the upper-right part of the graph clusters the eastern samples, which are very similar to each other, in spite they are

scattered over a half of the whole range of the species. This group corresponds to the subspecies *S. s. ligusticus* (Stefani, 1969), being the *terra typica* of this taxon Valdetaro Cave, near Rapallo (sample 11). A second group was represented by the westernmost populations (samples 22-29), including the samples (22-23) so far described as *S. s. bonzanoi* (Bruno & Bologna 1973) and *S. s. strinatii* (Aellen, 1958) (samples 24-29). The samples belonging to the subspecies *S. s. argentatus* (Stefani, 1969) (samples 19-21) and those belonging to a nameless form (15-18) were split in two differentiated groups. The first group (samples 19-21) was close to *S. s. ligusticus*. The second group was in the lower part of the graph and includes the Ligurian populations from the "Finalese" area (samples 15-18). These four populations are the most genetically differentiated in spite they are located in the central part of the species range. Therefore, PCA indicates that three main population groups could be found within *S. strinatii*: western populations (22-29), samples from "Finalese" (15-18), and eastern populations (1-14 and 19-21).

Table 3 - Genetic distances calculated according to Rogers' method (WRIGHT, 1978) for the three population groups identified within *S. strinatii*. Mean values below the diagonal, range of distances above the diagonal. On the diagonal are the ranges within each group.

Population group	"Eastern"	"Finalese"	"Western"
"Eastern" (samples 1-14,19-21)	0.008 – 0.212	0.251 – 0.374	0.196 – 0.375
"Finalese" (samples 15-18)	0.312	0.068 – 0.174	0.205 – 0.381
"Western" (samples 22-29)	0.247	0.292	0.108 – 0.344

The genetic distances calculated according to Rogers' method (WRIGHT 1978) supported the identification of three population groups (Table 3). The eastern samples (1-14 and 19-21) are shown to have a quite low genetic differentiation, with the easternmost populations (samples 1-14) showing very low D_{Rogers} (between 0.008 and 0.141), that slightly grew to 0.127-0.212 when the three samples from the central part of the range (19-21) are added. The populations from western range (samples 22-29) are well differenti-

ated both from the other population groups (D_{Rogers} between 0.196 and 0.381) and among them (ranging 0.108-0.344), confirming the genetic heterogeneity of this cluster. Finally, the populations 15-18 from “Finalese” are confirmed as highly differentiated, showing the highest recorded average distance from the other populations (average D_{Rogers} 0.312).

Table 4 - Mean genetic distances (D_{Nei} , NEI, 1972, below the diagonal) and divergence times in years (t , NEI, 1975, above the diagonal) between the three genetically characterised forms identified within *S. strinatii*.

Population group	“Eastern”	“Finalese”	“Western”
“Eastern” form (samples 1-14,19-21)	-	$t = 590.000$	$t = 375.000$
“Finalese” form (samples 15-18)	$D_{\text{Nei}} = 0.118$	-	$t = 500.000$
“Western” form (samples 22-29)	$D_{\text{Nei}} = 0.075$	$D_{\text{Nei}} = 0.100$	-

DISCUSSION AND CONCLUSIONS

The results obtained allowed the identification of three genetically and geographically characterised groups of populations. A genetically homogeneous group occupies a wide area (samples 1-14 from the eastern part of the range) and is genetically close to the population group from the western area (19-21). The relatively small westernmost part of the range hosts populations (22-29) that are highly differentiated both among them and from the other clusters identified. Finally, the most divergent population group is located in the central part of the range and covers a very small area in the “Finalese” region (samples 15-18).

From a taxonomic point of view, it was hard to find out a correspondence between these three groups and the formally described morphological subspecies. The “eastern” group (1-14 and 19-21) includes the *terra typica* of the subspecies *S. s. ligusticus* (Stefani, 1969), but also that of *S. s. argentatus* (Stefani, 1969) (cave Grotta delle Ruccaie, near Toirano, sample 19), along with the populations from Roburent area, usually included in this last subspecies. In the westernmost group are clustered all together the populations classified as *S. s. strinatii* (Aellen, 1958) (with the *terra typica*: the cave

Grotte d'Aspremont, n. 29) and *S. s. bonzanoi* (Bruno & Bologna, 1973) (samples 22 and 23, with this latter as the *terra typica* from the cave Tana I^a du Casà). The most genetically differentiated cluster from the Finalese area does not include any *terra typica*. These results clearly indicate that the so far proposed subspecies are inconsistent with the genetic structure of the species.

The genetic subdivision of *S. strinatii* in the three above identified population groups was consistent with the paleoclimatic history and the orography of the species' range. It is known that the speciation process in *Speleomantes* occurs mainly by divergence in allopatry, as in the larger part of plethodontids (GARCÍA-PARÍS *et alii* 2000), and is strongly influenced by paleoclimatic events via the rising of geographic barriers allowing the differentiation via mutation sum (FORTI *et alii* 1998; CIMMARUTA *et alii* 1998). In particular, it has been speculated that the glaciations played an important role at the evolutionary level, allowing the origination of the extant species by means of the alternation of glacial and interglacial periods during Quaternary (FORTI *et alii* 1998).

To test for the influence of glacial events on the genetic structuring of *S. strinatii*, the genetic distances between the so far identified groups of populations have been used to estimate the divergence times among them, according to Nei's calibration (NEI 1975). The obtained times have been compared with the paleoclimatic events occurred in the area, in particular with glacial/interglacial stages, in order to determinate if glacials can be identified as subdividing events, promoting population fragmentation and producing the extant articulated genetic structure of the species. These results are reported in Tab. 4.

The divergence of the most differentiated group from the Finalese area started during glacial stages: during Günz with respect to the "eastern" group (about 590.000 ya) and during Mindel (about 500.000 ya) with respect to the "western" group. In the Mediterranean area, the glacial stages were characterised by a dry and cold climate, while the interglacials had a more mild and humid climate (WEST 1969; BERTOLANI-MARCHETTI 1985; PIGNATTI 1994). As a consequence, arboreal vegetation and animal species were restricted in warmer refugia at low altitude and along coastal areas during the glacials. The populations from the Finalese live in a small area,

completely surrounded by relatively high mountains (Mt. Settepani, Mt. Carmo, Mt. Beigua), that have probably acted as efficacious geographic barriers during ice ages, causing isolation and differentiation of the local remnant population group.

The divergence of the two most far apart population groups (westernmost vs. easternmost) cannot be reconnected to a glacial stage. It is dated 350-400.000 years ago, at an interglacial stage, when higher altitude sites were colonised due to the range expansion of the arboreal vegetation. The genetic pattern of the “eastern” population group suggests a relatively recent range expansion, as witnessed by its high genetic homogeneity. Moreover, the close similarity with the populations from the Roburent area (samples 19-21), suggests an Apennine colonisation route from Emilia and Tuscany to western Liguria. The slightly higher genetic variability observed in the Roburent samples could be due to the “secondary contact” between the re-established populations invading the area and those resident in the Maritime Alps.

This scenario is supported by the observed pattern of allele frequencies in the populations from Roburent area, holding “western alleles” together with the more common “eastern alleles”. Moreover, the Maritime Alps are a well known suture zone for either plant and animal species and the depicted pattern has been proposed for many other taxa (KROPF *et alii* 2002 and references herein). This scenario could explain the impossibility of dating the divergence time between easternmost and westernmost population groups, being the data affected by the subsequent contact and gene flow between these two forms.

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ABSTRACT

The genetic structure of the plethodontid salamander *Speleomantes strinatii* (Aellen, 1958) has been analysed by means of allozyme electrophoresis. The studied samples were taken from the whole range of the species (north-western Italy and south-eastern France), to assess the degree and the pattern of genetic differentiation and to find out possible correlation with the paleoclimatic history of the area. The results obtained showed three genetically characterised population groups, whose distribution and levels of differentiation were related to the quaternary alternation of glacial and interglacial stages in the region. The subdivision of this species in four morphological subspecies is not supported by the genetic structure here evidenced.

RIASSUNTO

Gli effetti delle glaciazioni quaternarie sulla struttura genetica di *Speleomantes strinatii* (Aellen, 1958) (Amphibia, Plethodontidae).

La struttura genetica del geotritone *Speleomantes strinatii* (Aellen, 1958) è stata analizzata mediante analisi elettroforetica di sistemi gene-enzima, su popolazioni campionate in tutto l'areale della specie. Il grado di eterogeneità inter- ed intrapopolazionale, i livelli di variabilità e le relazioni di affinità genetica tra le varie popolazioni sono stati confrontati con la storia paleoclimatologica dell'area per configurare i possibili processi microevolutivi che hanno originato l'attuale struttura genetica della specie. I risultati ottenuti hanno evidenziato la presenza di tre gruppi di popolazioni geneticamente ben caratterizzati, la cui origine è probabilmente riconducibile all'influenza dell'alternarsi dei periodi glaciali ed interglaciali del Quaternario. I dati ottenuti sono stati anche utilizzati per verificare la validità delle sottospecie morfologiche attualmente riconosciute, che non risultano essere sostenute da basi genetiche ed evolutive.